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THE REMARKS

The Amendments

Claims 1, 2, 4-7, 9, 12-14, 18-21 and 23-27 are pending in the present application.

The specification is amended to provide the sequence identifiers for the nucleotide sequences and amino acid sequences.

Please amend Claims 1 and 4 and cancel Claim 29.

Claims 1 and 4 are amended to substitute " a polar amino acid" with "a serine". Support for the amendment is found, for example, in page 2, line 13.

Claim 4 is amended to recite "the mutated cDNA obtained in d)" by replacing "c)" with "d)". This amendment is to correct an obvious typographic error.

Claim 29 is canceled as amended Claim 1 is identical to Claim 29.

The above amendments are made solely in order to expedite prosecution of the application. Applicants reserve the right to file the original claims in one or more continuation-type application.

No new matter is added in any of the above amendment and the Examiner is respectfully requested to enter the amendments and reconsider the application.

The Response

1. **Objection Under 37 C.F.R. § 1.821(d)**

Applicants have amended the specification to identify the SEQ ID Nos. of the nucleic acid and amino acid sequences for Figures 2, 3A and 3B. The Brief Descrition as amended refers to the nucleic acid and amino acid sequences for Figures 2, 3A and 3B. Therefore the present application fully satisfies the requirements of 37 C.F.R. 1.821(d), and the Examiner is requested to withdraw this objection.

2. **Rejection Under 35 U.S.C. § 112, second paragraph**

(A) The Examiner rejects Claims 4-7, 9, 12-14 and 19-21 as allegedly being incomplete for omitting essential steps, such omission amounting to a gap between the steps. The Examiner alleges that the omitted steps are: "the step that results in the serine found at

position H100A of the product set forth in claims 1" (page 2, line 26).

As suggested by the Examiner, Applicants have amended Claims 1 and 4 to replace "a polar amino acid" with "a serine". Therefore, Applicants respectfully request the Examiner withdraw this rejection.

(B) The Examiner rejects claims 5-7, 9, 12-14 and 19-21 as allegedly lacking antecedent basis for the recitation "the mutated cDNA obtained in c)".

As suggested by the Examiner, Applicants have amended Claim 4 to recite "the mutated cDNA obtained in d)". Therefore, Applicants respectfully request the Examiner withdraw this rejection.

3. **Rejection Under 35 U.S.C. § 103(a)**

The Examiner rejects Claims 1, 4-6, 9, 12, 19-20, 23-25 and 28-29 under 35 U.S.C. §103(a) as allegedly being unpatentable over Kroon, *et al.* (*Pharmaceutical Res.* 9:1386-1393 1992) in view of Senoo, *et al.* (U.S. Patent No. 5,852,177) and Kipriyanov, *et al.* (*J. Immunol. Meth.* 196:51-62, 1996). Applicants respectfully traverse this rejection.

To establish a *prima facie* case of obviousness, there must be a reasonable expectation of success. The teaching or suggestion to make the claimed combination **and** the reasonable expectation of success must **both** be found in the prior art, not in the applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

The Examiner contends that "one of ordinary skill in the art . . . armed with the teachings of Kroon et al., would have been motivated to change the cysteine at position H100A (Kabat numbering system) in CDR3 of the OKT3 heavy chain by site-directed mutagenesis." (page 4, lines 17-19).

The Examiner responds to Applicants' arguments, that Kroon, *et al.* teach away from the substitution of the cysteine at H100A because the significant deleterious impact that it may have in the binding affinity of the antibody, by stating:

> ". . . the teaching of Kroon, et al., taken for all the teach [sic], provide clear guidance and motivation with respect to the replacement of the cysteine in CDR3 of OKT3."

(page 6, 5th paragraph).

The Examiner also responds to Applicants' arguments that there was no reasonable expectation that the substitution of cysteine with a serine would result in an antibody with almost no loss in binding affinity by contending that:

> "However, the instant claims do not recite any requirements regarding the binding affinity of the antibody. In addition, even were limitations regarding binding affinity recited, since there is sufficient motivation and reasonable expectation regarding the production of the same antibody product as that claimed, there would be no difference in the binding affinity." (page 6, 7th paragraph).

Applicants respectfully disagree with the Examiner's arguments. A reasonable expectation of success is an essential element of establishing a *prima facie* case for obviousness. Applicants respectfully contend that the Examiner has not demonstrated that any of the cited prior art teach or suggest that by substituting the H100A cysteine of the OKT3 antibody with serine there would be a reasonable expectation of success of producing an antibody with increased stability and sufficient binding affinity. Merely increasing the stability of an antibody would not result in a useful antibody if there were a significant loss of binding affinity.

Besides the stability of the antibody, the affinity and specificity are also essential for the functionality of the anti-human CD3 antibody. Both affinity and specificity of an antibody are determined to a large extent by the amino acid sequence of the CDR3 of the heavy chain (CDR-H3). One of ordinary skill in the art would know this and would try to retain the amino acid composition of the CDR-H3. For example, in all humanized variants of OKT3, the cysteine in CDR-H3 was retained (*see* Adair, *et al.*, 1994, and Woodle, *et al.*, 1992; references 1 and 14, respectively, cited in the PTO Form 1449 filed November 21, 2001). Further examples showing the cysteine in position H100A was retained are when recombinant OKT3 antibody was generated and expressed in CHO cells (Cole, *et al.*, *J. Immunol.* 159:3613, 1997; a copy of the abstract is attached) and also when recombinant bispecific anti-CEA/anti-CD3 diabody was generated (Fitzgerald, *et al.*, *Protein Eng.* 10:1221, 1997; a copy of which is attached). It is clear to one of ordinary skill in the art that, if the resultant recombinant antibody has increased stability but a total or significant loss of binding affinity, then the

substitution itself, to achieve the increased stability, defeats the purpose of having an increased stability. The Examiner has not provided any disclosure or teaching to demonstrate that it would be reasonable to expect that such a substitution would not result in an antibody with a reduced binding affinity. In fact, we again cite Kroon, *et al.* which predicts that such a substitution "may have a significant impact on the binding affinity of the antibody" (page 1390, left col., 2d paragraph). By "significant impact", one of ordinary skill in the art must understand that Kroon, *et al.* mean a "significant **negative** impact".

There is no reasonable expectation of success that substitution of Cys_{105} of the CDR-H3 of OKT3 (Kroon, *et al.*, page 1390, left col., 2d paragraph) with a serine would result in an antibody that is desirable merely because it may be more stable. The art at the time of filing the present invention includes the disclosure of Schier, *et al.* (*J. Mol. Biol.* 263:551, 1996; a copy of the abstract is attached). Schier, *et al.* (reviewed in Adams, *in vivo* 12;11-22, 1998) demonstrates that substituting the cysteine in the CDR-H3 of the C6.5 antibody with a serine results in a drastic reduction of the binding affinity from 1.0×10^{-7} M to 7.0×10^{-4} M (Kd); *i.e.* nearly a **1,000-fold reduction** of binding affinity (Adam, page 16, Table II and page 18, left col., 3rd paragraph). Such a drastic reduction of affinity would in fact teach away one of ordinary skill in the art from the making of a similar cysteine to serine substitution in the third CDR region of another antibody. This disclosure is consistent with the disclosure of Kroon, *et al.*, which also teach away from the substitution of the cysteine at H100A of the V_H domain of the OKT3 antibody. As Applicants have previously pointed out in the Response filed June 20, 2003, changing the CDR of an antibody may negatively impact the binding affinity of such an antibody.

Therefore, one of ordinary skill in the art, at the time this application was filed, would not have a reasonable expectation of success of substituting the cysteine at H100A of the V_H domain of the OKT3 antibody to produce a recombinant antibody of increased stability and sufficient binding affinity to CD3. Since the cited references do not render Claims 1, 4-6, 9, 12, 19-20, 23-25 and 28 obvious, Applicants respectfully request the Examiner withdraw this rejection.

The Examiner rejects Claim 26 under 35 U.S.C. §103(a) as allegedly being unpatentable over Kroon, *et al.* in view of Senoo, *et al.* and Kipriyanov, *et al.*, and in further view of Nitta, *et al.* (*The Lancet* 335:368-71, 1990). Applicants respectfully traverse this rejection.

For the reasons provided earlier, Kroon, *et al.*, Senoo, *et al.* and Kipriyanov, *et al.* do not render obvious an antibody comprising the V_H domain of the antibody produced by the hybridoma of ATCC deposit number CRL 8001, wherein the cysteine at position H100A of said V_H domain is substituted with a polar amino acid, or a method of producing the antibody.

Adams (*in vivo* 12:11-22, 1988) in reviewing Schier, *et al.*, shows that further results of amino acid substitutions at the cysteine residue in the CDR-H3 region of the C6.5 antibody. Of thee four amino acid substitutions recorded (page , Table II), substitutions with a polar amino acid residue (serine) had a significantly greater negative impact o affinity than the other substitutions which used a non-polar amino acid residue (valine or alanine) or a basic aminoa cid residue (lysine).

The addition of Nitta, et al. does not overcome the deficiency of Kroon, *et al.*, Senoo, *et al.* and Kipriyanov, *et al.*; as Nitta, *et al.* also do not teach that substituting the cysteine at the H100A position with a polar amino acid would result in an antibody with an increased stability **and** with almost no loss of its original binding affinity.

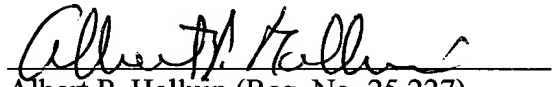
Since the cited references do not render Claim 26 obvious, Applicants respectfully request the Examiner withdraw this rejection.

CONCLUSION

In view of the foregoing amendments and remarks, the Applicants believe the application is in good and proper condition for allowance. Early notification of allowance is earnestly solicited. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is encouraged to call the undersigned at (650) 463-8109. A telephone conference is especially requested if the Examiner intends to maintain the present rejections.

Respectfully submitted,

Date: February 5, 2004



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in vivo 12: 11-22 (1998)

Improving the Tumor Specificity and Retention of Antibody-Based Molecules

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Abstract. *A number of novel strategies have been employed to enhance the degree and specificity of tumor-targeting by antibody-based molecules. These fall into three overlapping categories. The augmentation of the interaction between the antibody and target antigen (e.g., through increased affinity or valence), the enhancement of targeting specificity (e.g., by altering the clearance of unbound antibody or radiolabel), and the selection of radioisotopes and labeling methods which are more stable in vivo or better suited to the target application.*

More than twenty years have passed since Kohler and Milstein's seminal report on the development of monoclonal antibodies (MAb) (1). The field of antibody-based therapeutics has progressed greatly over this period, as evidenced by the recent reports of successful treatment of hematological malignancies by a number of groups (2, 3). However, advances in the application of MAb for the treatment of solid tumors have been greatly limited by tumor physiology (4). The disordered vasculature and lack of draining lymphatics in tumors lead to elevated interstitial pressure which in turn limits the intratumoral diffusion of an IgG molecule to about one millimeter in two days. Identification of these obstacles has fostered new strategies for increasing tumor-specific targeting by antibody-based molecules. These include size modification or the addition of side groups to alter the rates of both systemic clearance and extravasation into tumor, the use of pretargeting strategies and the optimization of radiolabeling methods (*e.g.*, residualizing labels or metabolizable linkers).

However, the largest improvements in the specificity of antibody-based targeting have stemmed from recent advances in the field of antibody engineering which have facilitated the production of novel antibody-based molecules (Figure 1). Efforts to minimize the size of proteins capable of recognizing antigen have led to the development of monovalent species ranging in size from 1 to 25 kd, while the employment of diverse dimerization strategies has created molecules with varying degrees of flexibility and avidity. Finally, site-directed mutagenesis and chain-shuffling have been used to enhance the affinity of the antibody-antigen interaction. This plethora of reagents has enabled investigators to begin to identify the features (*e.g.*, size, orientation and flexibility) required for optimal tumor targeting.

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Key Words: Affinity, tumor targeting, monoclonal antibodies, single chain Fv, radiolabeling.

Engineered antibody-based molecules

Many of the limitations of hybridoma-based technology have been recently overcome through the humanization of murine MAb (reviewed in 5), the creation of single-chain Fv (scFv) fragments (6, 7) and the development of large combinatorial immunoglobin phage display libraries (8). It is now possible to generate human IgG or scFv which can likely be administered repetitively to patients without eliciting an immune response. Furthermore, by selecting from large combinatorial libraries, antibodies can be generated with a specificity for virtually any antigen, including those that are highly conserved (9). The cloning of the immunoglobulin genes into a plasmid then allows for expression in *E. coli* and provides an environment in which rational modifications can be readily accomplished. The following antibody-based species appear to possess the greatest potential to alter the specificity and degree of tumor retention. Descriptions of their *in vivo* properties, where available, are provided in detail below.

Complimentarity determining regions. Sivolapenko *et al* have produced a 15 amino acid synthetic peptide derived from the heavy chain complimentarity determining region #3 (CDR3) of the ASM2 mouse IgG1 specific for the MUC-1 antigen, a 20 amino acid tandem repeat expressed in greater than 90% of epithelial carcinomas (10). As the heavy chain CDR3 frequently mediates the dominant interaction between an antibody and its target epitope, this likely represents the smallest possible antib dy-derived molecule with a specificity

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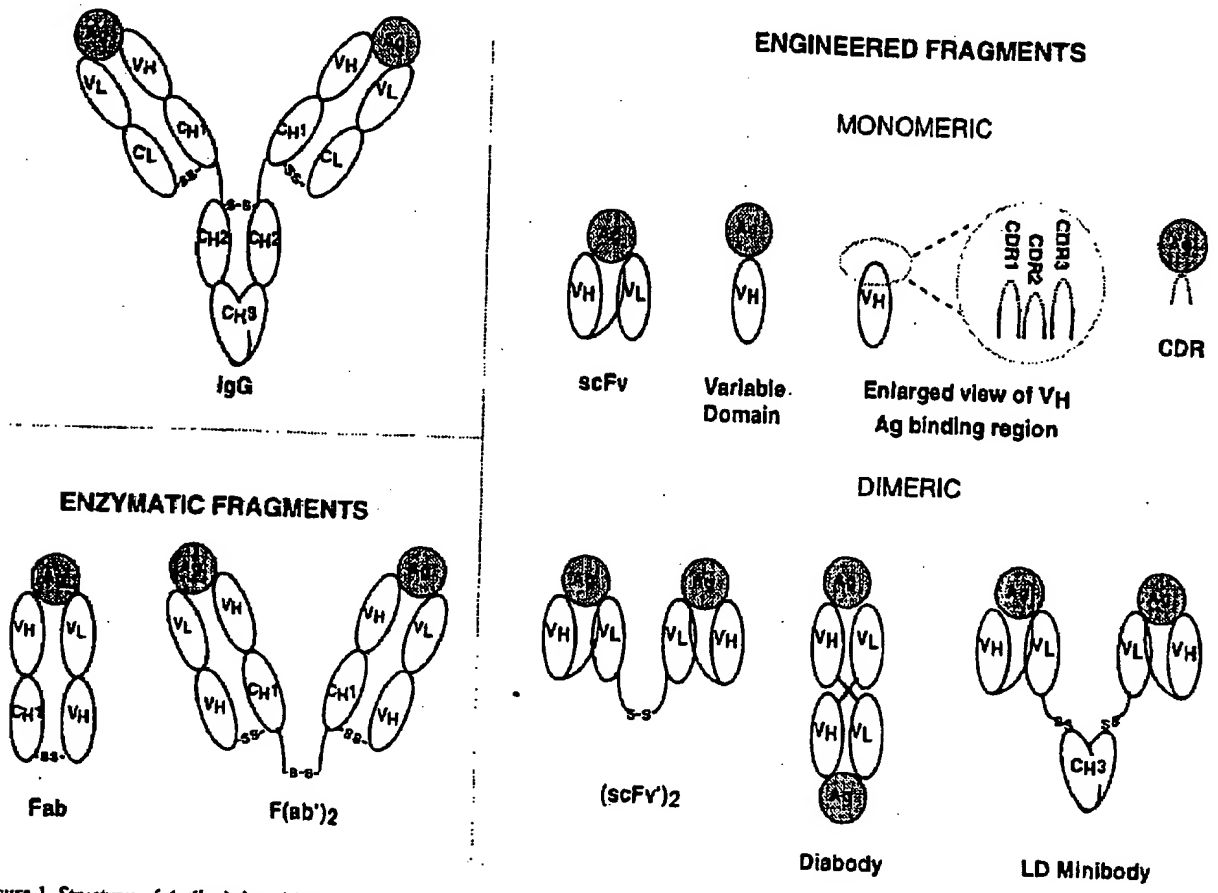

Figure 1. *Structures of Antibody-based Molecules. Schematic diagram showing the structures of antibody-based molecules. The parent IgG molecule can be enzymatically digested to yield Fab or F(ab')2 fragments. Alternatively, using recently developed antibody engineering techniques, a variety of novel monomeric and dimeric molecules have been developed.*

for a tumor antigen (Figure 1). However, by reducing the antibody/antigen interaction from 6 CDRs to a single CDR, the likelihood of crossreactivity with other unrelated antigens increases significantly. Furthermore, the increased flexibility of th unsupported peptide limits its interaction with epitopes requiring spatial conformations.

Variable domains. Independent variable heavy (V_H) or variable light (V_L) domains (12kd) contain one half of an antibody's antigen binding site (Figure 1) (11). Variable domains can be expressed from *E. coli* or mammalian cells. Unlike the single CDRs described above, intact variable domains contain both the antigen contact residues and the supporting framework domains. This allows binding to conformational epitopes and potentially reduces the incidence of crossreactivity with unrelated epitopes.

Single-chain Fv molecules. Single-chain Fv (scFv) molecules are 25 kd proteins composed of peptide-linked V_H and V_L domains (Figure 1). They can be produced from genes isolated from hybridomas expressing MAb of a desired specificity (6, 7) or can be isolated by panning (selection) from a combinatorial phage display library (9). As scFv contain the entire antigen binding domain, their specificity and affinity are typically equivalent to that of an intact MAb. However, in settings in which binding avidity is important, scFv are limited by their monovalent nature.

$(scFv')_2$. Created by producing scFv with carboxy-terminal cysteine residues, these 55 kd dimeric proteins have a moderate degree of flexibility and a relatively compact shape (Figure 1) (12). This structure facilitates divalent binding of targeted antigen without excessively limiting the ability to penetrate solid tumors.

Diabodies. Diabodies are produced by reducing the length of the peptide linker between the V_L and V_H domains of an scFv to prohibit pairing between domains of the same chain. As a consequence, pairing occurs between complementary domains of two different chains, creating a stable, n n-

Table I. *A survey of the potential antibody-based molecules reported in the literature.*

Molecule	Size	Valence	Specificity	$T_{1/2}\alpha$	$T_{1/2}\beta$	Ref-
CDR3		1		3.6 min[a]	0.9 h[a]	(10)
V_H	12 kd	1				(11)
scFv	25 kd	1	HER2, CEA, Tag-72	2.4-12 min	1.5-3.9 h	reviewed in (62)
diabody	50 kd	2	HER2	40 min	6.4 h	(23)
$(scFv')_2$	55 kd	2	HER2	13 min	2.4 h	(22)
Fab	50kd	1	Numerous	9.1 min	1.5 h	(17)
Miniantibody (scdHLX)	64 kd	2	phosphocholine	17.5 min	3.4 h	(63)
Miniantibody (scZIP)	64kd	2	phosphocholine	11.9 min	4.1 h	(63)
FlexMinibody $(scFv)_2$-CH3	80kd	2	CEA	35.2 min	5.3 h	(14)
LD Minibody (scFv)2-CH3	80 kd	2	CEA	72.6 min	4.8 h	(14)
$F(ab')_2$	100kd	2	Numerous	0.4 h	6 - 12 h	(17, 26)
Fab/c	105 kd	1			64 h	(65)
IgG	150 kd	2	Numerous	0.7 - 2.6 h	50 - 113 h	(17, 22, 26)
$F(ab')_3$	152 kd	3	CEA	n.a.	n.a.	(46)
$(IgG)_2$	300 kd	4	Le^y	n.a.	26 h	(45)
IgM	970kd	10	Numerous	n.a.	n.a.	(27, 64)

[a]Pharmacokinetics determined in human patients.

covalently associated dimer with two functional binding sites (Figure 1) (13). The compact and inflexible nature of these 52 kd proteins likely limits their utility to targeting epitopes which are relatively accessible.

Minibodies. Larger divalent molecules, termed minibodies, are produced by fusing the gene for an scFv to that for a human IgG1 C_H3 domain (Figure 1) (14). Dimerization of the resulting proteins occurs spontaneously, mediated by the high affinity (10^{10}- 10^{12} M^{-1}) interaction between two C_H3 domains (15). The minibody has been produced in the "Flex" and "LD" formats. In the Flex minibody, a flexible extended human IgG1 hinge region is used to connect the V_H and C_H3 domains, while in the LD minibody, the domains are connected by a more restrictive two amino acid spacer. These 80 kd minibodies are the only engineered antibodies in this group which may be expected to exceed the renal threshold for first pass elimination on the basis of molecular weight (discussed below).

Altered clearance and tumor penetrance

Size modification. An examination of the reported pharmacokinetics and biodistribution of the engineered molecules, intact immunoglobulins and enzymztically prepared immunoglobulin fragments in tumor-bearing mice reveals an association between size, systemic clearance and tumor penetrance (Table I). The pharmacokinetics, or blood clearance, of these proteins depends primarily upon their size in relation to the renal threshold and their charge (reviewed in 16). In general, uncharged proteins of less than 20 A in size can pass through the glomerulus without restriction while those greater than 40 A are effectively restricted. The addition of charge further alters clearance through the kidneys (discussed in greater detail below). For antibody-based molecules, this generally results in a very rapid clearance of constructs with a molecular weight less than approximately 65 kDa and a prolonged retention of species exceeding 65 kDa. Accordingly, the smallest molecules (*e.g.*, CDRs and variable domains) exhibit the fastest half-life in the circulation. In patients, fifteen amino acid peptides derived from the heavy chain CDR3 were rapidly eliminated from the circulation in a biphasic manner, with an equilibration half-life ($t_{1/2}\alpha$) of 3.6 minutes and an elimination half-life ($t_{1/2}\beta$) of 54.1 minutes (10). Escalations in administered dose did not affect the pharmacokinetic or distribution patterns. Overall, 14 of 15 primary tumors and 8 of 8 local recurrences were successfully visualized, often as early as 30 minutes after

administration. The incidence of false positives was not addressed and could be a major problem as the likelihood of binding to unrelated non-tumor antigens is increased by the dependence upon the specificity dictated by a single CDR. The extremely small size and rapid systemic clearance of this class f molecules limits the quantity and durati n of tumor localization. Accordingly, single CDRs are unlikely candidates for the radioimmunoimaging or therapy of cancer. While there is no report of pharmacokinetics of solitary V_H or V_L domains in humans or immunodeficient mice, they would likely be associated with deficiencies similar to those reported for CDRs. Again, the incomplete binding domain and low molecular weight are expected to result in both an increased potential for reacting with unrelated antigens and a rapid systemic clearance.

Unlike the CDR peptides and variable domain fragments described above, scFv molecules contain an intact binding domain and thus exhibit specificities and affinities that are similar to those reported for their parent MAbs (12, 17, 18). Radiolabeled forms of these molecules typically are associated with the highly specific tumor localization of small quantities (*e.g.*, less than two percent of the injected dose) in their terminal distribution phase. This retention pattern has proven to be ideal for radioimmunoimaging studies performed in mice (12, 14, 17) and patients (19).

While the small size of the scFv enables tumor penetration superior to that achieved with larger fragments or intact IgG (20), its half-life in circulation is still too rapid to allow sufficient localization for therapeutic effects. Unless major modifications (discussed below) can be made to either the systemic clearance or the tumor retention of scFvs, their utility will likely be limited to imaging applications.

The next largest group of antibody-based molecules are the Fab fragments, engineered $(scFv')_2$ and diabodies. With masses in the range of 50-55 kDa, these molecules are eliminated from circulation slower than the smaller species described above. However, as they are expected to fall beneath the renal threshold, their clearance is rapid as compared to the larger molecules described below. Despite their similar sizes, the three structures exhibit markedly different kinetics and tumor retention properties when assayed *in vivo*. These differences possibly result from their disparate globular structures (Figure 1), and may be indicative of dissimilar tumor penetrance and renal elimination properties. We have examined the distributions of radioiodinated 741F8 Fab, 741F8 $(scFv')_2$ and C5.6 diabody molecules, all specific for the HER2/*neu* antigen which is overexpressed in a high percentage of breast, ovarian and colon cancers. While the 741F8 and C6.5 molecules target different epitopes of HER2/*neu*, their scFv forms exhibit very similar pharmacokinetics ($t_{1/2}\alpha$, 12 minutes, 3.9 hours) and 24-hour tumor retentions (approximately 1% ID/g) in *scid* mice bearing s.c. human SK-OV-3 tumors overexpressing the HER2/*neu* antigen (12, 21) (and unpublished results). However, the 741F8 $(scFv')_2$ was bserved to have pharmacokinetics ($t_{1/2}\alpha$, 13 minutes, $t_{1/2}\beta$, 2.4 hours) which were faster than its scFv form (22), and significantly faster than those of the C6.5 diabody ($t_{1/2}\alpha$, 40 minutes, $t_{1/2}\beta$, 6.4 hours) (23). This resulted in greater 24 hour tumor retention of the C6.5 diabody (6.5% ID/g) (23) than was observed with 741F8 $(scFv')_2$ (1.6% ID/g) (12) or 741F8 Fab (1.3% ID/g) (12). Still, the tumor retention of all three molecules remained well below that achieved with 741F8 IgG (20% ID/g) (22), albeit with greater targeting specificity conferred by their rapid systemic clearances. The highly specific nature of the tumor retention of C6.5 diabody in this model led to cumulative tumor and organ dosimetry predicted to approach therapeutic levels when high energy isotopes are employed. In fact, in a preliminary radioimmunotherapy study we performed in nude mice with a single dose of 1.2 mCi of ^{131}I-C6.5 diabody, the growth rate of large, established, radiation-insensitive SK-OV-3 tumors was decreased by 50% and duration of survival was nearly doubled, as compared to untreated controls (unpublished data).

As discussed above, 80 kDa minibodies are expected to exceed the renal threshold for first pass elimination. As such, they exhibit significantly prolonged pharmacokinetics in immunodeficient mice ($t_{1/2}\alpha$, 35 minutes, $t_{1/2}\beta$, 5.3; for the Flex minibody) as compared to that observed with smaller scFv (14). Interestingly, while the $t_{1/2}\beta$ of the Flex and LD forms of the minibody were very similar, the LD minibody exhibited an equilibration phase ($t_{1/2}\alpha$) in blood that was more than twice that of the Flex minibody. It was unclear if the accelerated accessibility to tumor or increased flexibility of the Flex form led to tumor retentions that were significantly greater than those reported for the LD form (29% ID/g *vs.* 8% ID/g, respectively at 24 hours). The similarities between the pharmacokinetics of the minibodies and the C6.5 diabody described above may indicate similar renal processing or elimination (Table I). As with the diabody, the slower elimination from circulation contributed to an increased retention in tumor. While the absolute tumor retention was greater with the Flex minibody than with the diabody, similar cumulative exposures to tumor and normal tissues were calculated for both molecules (*e.g.*, tumor :blood area under the curve ratios of 4.5:1 [flex minibody] and 3:1 [diabody]), again suggesting a similar processing.

Larger, naturally-occurring and enzymatically derived molecules such as IgM, IgG and $F(ab')_2$ have been described in detail elsewhere and therefore will not be extensively reviewed here (reviewed in 24, 25). Of this group the 100 kDa $F(ab')_2$ fragments exhibit the most promising combination of relatively rapid pharmacokinetics ($t_{1/2}\alpha$, 0.4 hour, $t_{1/2}\beta$, 6-12 hours) and quantitative tumor retention (20% ID/g at 24 hours) (17, 26). While larger 150 kDa IgG molecules display high, durable levels in tumor (>20% ID/g) for up to a week post injection, they rarely achieve significant targeting specificity (17, 22). Finally, extremely large, 970 kDa IgM molecules are succeptable to aggregation, resulting in rapid clearance by the liver and very poor tumor uptake (27).

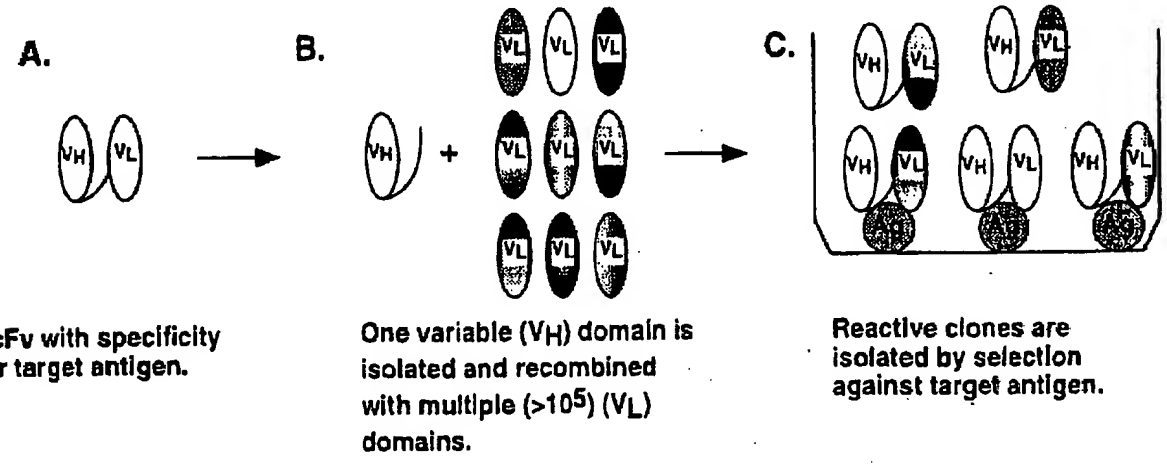

Figure 2. *Chain Shuffling. Increases in affinity can be achieved by chain shuffling. In this process which is outlined in the figure, one variable chain (light or heavy) is retained to maintain antigenic specificity while the other chain is removed and replaced with numerous other variable chains from a phage display library (B). Reactive clones are then isolated by selection (panning) against the target antigen (C). This recombination typically yields many scFv with reduced or ablated affinity for the target antigen and a handful of scFv with increased affinity.*

The impact of molecular weight on the tumor penetrance of antibodies and their fragments was elegantly demonstrated by Buchegger *et al* in an anephric mouse model (28). By ligating the kidneys, rapid excretion of the antibody fragments was prevented, allowing an evaluation of the role of size on tumor uptake in a setting where steady state levels were maintained in circulation. In this model, the investigators reported that at 8 hours following administration the lowest accumulation in tumor was observed with the intact MAb, and that the tumor retention of the $F(ab')_2$ and Fab fragments were 1.4 and 2 times greater than the MAb, respectively. This indicates that smaller, engineered antibody-based molecules have the potential to be superior agents for tumor targeting if their systemic clearance can be delayed or retarded.

The information reviewed above suggests an obvious correlation between the size of a molecule and its tumor targeting, tumor penetrance and pharmacokinetic properties. Clearly, the smallest molecules (*e.g.*, CDRs and probably variable domains) are cleared at such a rapid rate that very little accumulation occurs in tumor. Somewhat larger scFv and $(scFv')_2$ exhibit highly specific retention in tumor, which is greater than that achieved with the smallest molecules, but insufficient for therapeutic applications. Currently, diabodies and minibodies appear to possess the best balance between degree and specificity of tumor retention. Their clearance from circulation is slow enough to allow significant accumulation in tumor without excessive retention in normal tissues, while their relatively small size should allow for effective tumor penetration. Accordingly these molecules may have the greatest potential as vehicles for radioimmunotherapy. Finally large IgG and IgM molecules have repeatedly been found accumulate at the highest levels in tumor, with the poorest penetration and targeting specificity. However, it is important to note the dangers inherent in the comparison of results acquired with different antibody-based structures targeting contrasting tumor antigens (shed *vs.* cell surface) in dissimilar tumor models. The head to head evaluation of molecules (particularly diabodies and minibodies) derived from a single antibody in a standard animal/tumor model is required for the generation of firm conclusions.

Side groups. The clearance rate and tumor diffusion of antibody-based molecules can be greatly altered by the addition of functional side groups or charge modification. As noted above, such strategies may prove to be useful in enhancing the therapeutic potential of the smaller, rapidly cleared molecules. Effective immunoconjugates have incorporated biological response modifiers (*e.g.*, IL2) or polyethylene glycol (PEG). When administered systemically, IL2 causes a capillary leak syndrome resulting in the accumulation of intravascular fluid in extravascular spaces. Localization of this effect to tumor has been achieved through the conjugation of IL2 directly to a $F(ab')_2$ fragment. In the initial studies performed by LeBerthon *et al*, the tumor retention with $F(ab')_2$/IL2 immunoconjugate at 48 hours post injection was four-fold greater than with $F(ab')_2$ alone and twice that achieved with co-injections of $F(ab')_2$ and IL2 (29). Furthermore, since the capillary leak associated with the immunoconjugate was limited to the tumor, the specificity of localization, as measured by tumor:organ ratios, was correspondingly enhanced. More recently, other biological

response modifiers such as GM-CSF and TNF have been employed in the construction of immunoconjugates and are currently under evaluation (30, 31).

The covalent attachment of PEG to peptides, cytokines and enzymes delays their elimination from circulation by a variety of mechanisms including an increased evasion of renal and/or cellular clearance, enhanced resistance to proteolysis, reduced immunogenicity and improved solubility (reviewed in 32). Accordingly, PEG-based modifications are particularly attractive for prolonging the circulation, and hence the bioavailability, of smaller antibody-based reagents like Fab fragments or scFvs. This is consistent with the report of Pedley *et al* in which IgG, $F(ab')_2$ and Fab' fragments of the anti-CEA antibody ASB7 were chemically conjugated to PEG (Mr 5000) and radioiodinated prior to evaluation in a biodistribution study in nude mice bearing human tumor xenografts. While the distribution of the modified IgG was similar to that observed with its unmodified form (44% ID/g *vs.* 42% ID/g, respectively in tumor at 24 hours), $F(ab')_2$-PEG and Fab'-PEG displayed significantly prolonged plasma half-lives and enhanced tumor localizations. Of particular interest was the overall doubling of the circulatory retention of the PEG-modified $F(ab')_2$ as compared to the unmodified $F(ab')_2$ fragment. As both molecules fall well above the renal thresh ld for first pass clearance (approx. M_r 70,000), this suggests that increased size resulting from PEG conjugation was not a significant factor. Furthermore, the lack of a similar magnitude of increase in normal tissue retention of the PEG-conjugated fragments led to an overall enhancement in tumor specificity.

Modification of the charge or isoelectric point (pI) of an antibody can change its interaction with cells, including those of the tumor, endothelium, and kidney. Cationization of a MAb, through the conversion of surface carboxyl groups to extended primary amino groups, was reported to dramatically increase the rates of both systemic clearance (8-fold faster than native) and endocytosis (7-fold greater than native) into targeted tumor cells (33). This was thought to be a function of an increased attraction between the positively charged MAb and the negatively charged tumor and endothelial cells. Conversely, the reduction of the pI or acetylation of an ^{111}In-labeled Fab' fragment led to both a reduction in the rate of clearance from circulation and the retention in the kidneys (34). This effect was believed to have resulted from repulsion between the negatively charged protein and the electronegative cells of the endothelium and the glomerular basement membrane, and is consistent with the observation that modification of the pI of albumin from 4.9 to 7.2-8.2 increased renal excretion by a factor of 300 (35). Interestingly, tumor uptake of the aceylated and unmodified Fab' fragments was comparable despite a 50% reduction in the ability of the aceylated Fab' to bind to tumor cells *in vitro*. This suggests that the increased retention in circulation compensated for the decreased ability to bind antigen. These studies indicate that charge modification can be rationally employed to alter the pharmacokinetics and targeting of antibody-based proteins.

Table II. *Site-directed Mutagenesis. An example of the processes employed for the affinity maturation of single-chain Fv molecules. Critical amino acids are identified by the substitution of alanine residues; (alanine scanning) at each position in the CDR. Positions which are associated with a decrease in affinity following the substution are randomized and the resulting clones are assayed for activity and affinity.*

Clone	VH CDR3 Sequence	Affinity (Kd)
A. Parent Molecule	HDVGYCSSSNCAKWPNYFQH	1.0×10^{-7} M
B. Alanine Substitution	-------- A --------	6.0×10^{-5} M
C. Random Mutagenesis of Critical Residue(s)	-------- K --------	2.0×10^{-8} M
	-------- V --------	30×10^{-6} M
	-------- S --------	7.0×10^{-4} M

Enhanced antibody/antigen interactions

While the affinity of an antibody for its target antigen clearly is important in dictating tumor-targeting properties; the optimal range of affinities has yet to be identified. Two philosophies are currently in vogue. The first, that "more is better", is based upon the concept that with greater affinity there will be increased extraction of the antibody from blood and a longer retention in tumor will result. The second theory, termed the "binding site barrier effect", was developed by Weinstein and predicts that antibodies of exceedingly high affinity will irreversibly bind to the first target antigen encountered in the tumor, resulting in a rimming or patching of antibody around the vasculature with little penetration or diffusion into tumor (36).

The theory that greater affinity yields enhanced tumor retention is supported by the preclinical observations by Colcher, *et al* in which higher affinity second generation MAbs specific for TAG-72 were compared with the lower-affinity first generation molecules (37). In this study, the second generation MAb CC49 ($Kd = 6 \times 10^{-11}$) localized human tumor xenografts in athymic nude mice better than the lower affinity first-generation molecule B72.3 ($Kd = 2 \times 10^{-9}$). However, in subsequent clinical trials, the higher affinity CC49 failed to display improved tumor retention over that observed for B72.3 (38). As the authors noted, it was unlikely that affinity was the only variable affecting the tumor localization properties. Other factors such as accessibility and quantity of the targeted epitopes may have influenced the results.

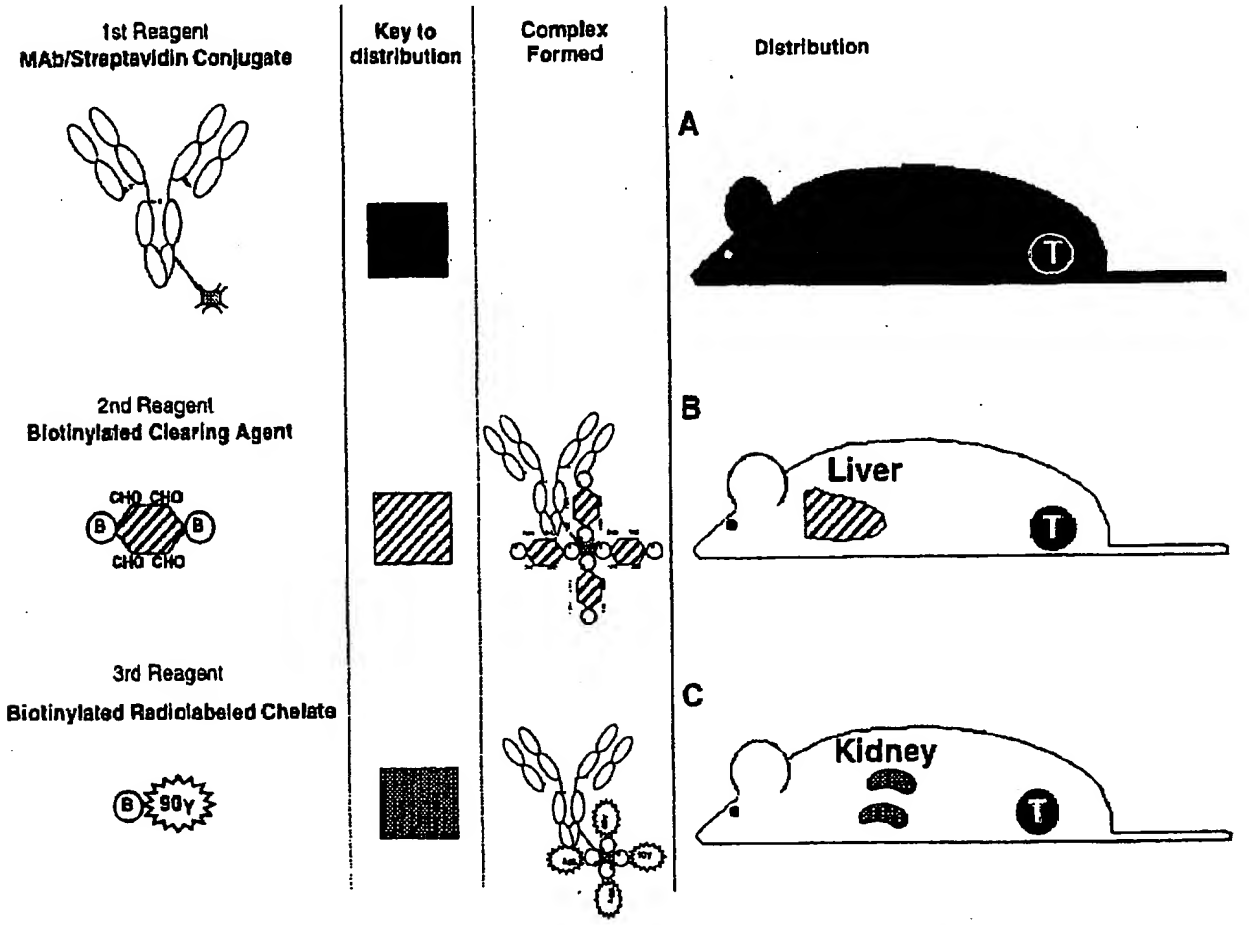

Figure 3. *Pretargeting. Schematic diagrams and a key to the in vivo distribution are shown for the molecules typically employed in pretargeting strategies. In the first step (A), the MAb-streptavidin conjugate is administered and distributes throughout the mouse, localizing to tumor (T) with poor targeting specificity. After approximately 24 h, biotinylated glycosylated albumin is administered which complexes with circulating MAb-streptavidin and is rapidly eliminated in the liver (B), leaving significant quantities of MAb-streptavidin in the tumor. After all of the biotinylated glycosylated albumin has left the circulation, the biotinylated radiolabeled chelate is administered. This molecule is quickly distributed throughout the animal and rapidly eliminated via the kidneys, with significant quantities remaining only in the tumor where it is "trapped" by the MAb-streptavidin.*

Support for the "binding site barrier effect" comes from observations comparing the distributions of the high affinity (Kd = 6.3 x 10^{-11}) MAb D3, specific for the chemically-induced L10 carcinoma line with a negative control antibody. In studies performed in guinea pigs bearing intradermal L10 tumors, administration of radioiodinated D3 MAb resulted in localization in peripheral antigenic patches, while the radioiodinated negative control MAb was uniformly distributed throughout the tumor (39). This indicated to the authors that the high affinity MAb was unable to penetrate deeply into the tumor. The difficulty in reconciling the results of these two studies arises from the diversity of the reagents and models employed. In both studies, affinity for the target antigen was not the only difference between the MAbs. In the former report, different epitopes of the TAG-72 antigen were targeted by CC49 and B72.3. It is therefore possible that epitope accessibility played a role in the observed differences. Furthermore, as noted above, differences in the physical properties (*e.g.*, pI) of the MAbs employed in each study could impact on their ability to extravasate into the tumor. Clearly a rigorous examination of this issue requires the employment of essentially identical MAbs with a range of affinities for an identical epitope.

Affinity modification. In a natural immune response the affinity of the polyclonal antibodies for their target antigen increases with serial exposure through a process known as affinity maturation (40). It has recently become possible to mimic this process for a single antibody binding site *in vitro* using chain shuffling (41) and site-directed mutagenesis (42).

These techniques allow the generation of extremely high affinity reagents from those with moderate affinities, while maintaining specificity for a given epitope. Thereby increasing the therapeutic potential of an scFv and providing a series of reagents to clarify the role of affinity in tumor targeting allowing for the identification of the optimal range of affinity for therapeutic applications.

Our efforts in affinity modification focused on the human anti-HER2/*neu* scFv C6.5. Originally isolated from a naive human phage display library with a repertoire of 107 specificities, the C6.5 scFv binds to the HER2/*neu* antigen which is overexpressed in a variety of cancer types with a moderate affinity (1.6×10^{-8} M) (21). Initial efforts to improve C6.5 employed the chain shuffling technique in which the heavy chain of the C6.5 scFv was recombined with new partner light chains from the phage display library in order to identify new pairings with a higher affinity for HER2/*neu* (Figure 2). After four rounds of selection, clones with up to 6-fold greater affinity were identified (43).

Significantly greater improvements in binding affinity were achieved through the site-directed mutagenesis of the C65 scFv heavy chain CDR3 region. For this process, critical CDR3 amino acid residues first were identified by sequential substitution with alanine residues and then were randomized in groups of three (42) (Table II). Finally, clones with affinities up to 1000-fold greater than that of C6.5 were achieved by combining two different heavy-chain CDR3 mutations with the best clone resulting from the light chain shuffling strategy. Interestingly, while we found that both the chain shuffling and site-directed mutagenesis methods led to improvements (reductions) in the rate of dissociation (k_{off}) of the scFv from antigen, the rate of binding to antigen (k_{on}) was not significantly altered.

Using the C6.5 scFv affinity mutants described above, we have recently initiated an investigation into the impact of affinity on the tumor retention of small scFv molecules. Working in scid mice bearing established human SK-OV-3 tumors overexpressing the HER2-*neu* antigen, we have found that sequential increases in affinity of the C65 scFv mutants from 3.2×10^{-7} M to 1.6×10^{-8} M to 1.0×10^{-9} M led to 4.2 and 7.5-fold increases in 24 hour tumor retention, respective to the lowest affinity variant (44). However, initial biodistributions performed with mutants with affinities above 10^{-9} M have failed to reveal continued increases in tumor retention (unpublished data). At present, it is unclear if these results stem from the rapid clearance of the small scFv molecules or if other factors such as the binding site barrier effect (36) or alterations in physiological properties (*e.g.*, pI or protein folding) are responsible.

Valence. The functional affinity, or avidity, of a molecule can also be enhanced by increasing its number of binding sites, or valence. Reports have been published on the construction and distribution of tetravalent IgG molecules (45) and trivalent F(ab')3 complexes (46). In both cases, increasing valence enhanced the performance of the antibody. Significant increases were observed in the ability of the tetravalent IgG molecule to slow the growth of s.c. tumors in mice, as compared to that seen with the divalent parental IgG molecule. While the tumor uptake of the trivalent $F(ab')_3$ complex exceeded that of a $F(ab')_2$ molecule and more closely mimicked intact IgG of approximately the same molecular weight. Similar increases in tumor retention are reported when dimeric scFv molecules are compared to their monovalent components (14, 47). However, in all of these studies, the increases in valency were linked to significant increases in molecular weight. Therefore it is unclear if the observed effect were due to changes in the number of binding sites or alterations in the systemic clearance, and hence the bioavailability of the molecule, resulting from the increased mass.

We have used two different approaches to address the role of valence on the binding of scFv-based molecules. The first was a direct comparison of the tumor retention of radioiodinated dimeric anti-HER2/*neu* 741F8 $(scFv')_2$ and enzymatically-prepared 741F8 Fab in *scid* mice bearing s.c. human SK-OV-3 tumor xenografts overexpressing HER2/*neu* (47). In these studies, the observation of significantly greater tumor retention with the $(scFv')_2$ as compared with the similarly sized Fab fragment suggested that increased avidity mediated enhanced tumor retention. However, potential differences in the globular structure of the $(scFv')_2$ and Fab molecules could have been responsible for the observed differences. Accordingly, we developed heterodimeric 741F8/26-10 $(scFv')_2$ molecules with the same overall structure as the dimeric 741F8 $(scFv')_2$, but with one binding site for the HER2/neu antigen and the second binding site (26-10) specific for an irrelevant antigen (digoxin) (48). When assayed in the tumor-bearing scid mouse model described above, three times more radioiodinated 741F8 $(scFv')_2$ localized in tumor than 741F8/26-10 $(scFv')_2$ (49). Furthermore, the tumor localization of the 741F8/26-10 $(scFv')_2$ heterodimer was indistinguishable from that observed with monovalent 741F8 scFv, supporting the theory that increased valence enhances antibody retention.

Radiolabel

Recently a greater emphasis has been placed upon tailoring a radiolabel to both the biological half-life and fate of an antibody. The importance of the former is illustrated by rapidly cleared scFv which are less efficient when paired with isotopes with long half-lives as the majority of the isotope's disintegrations will occur after renal elimination, while the latter is apparent when antibodies radioiodinated by traditional methods (*e.g.*, chloramine T) lose their labels following the internalization into tumor cells. A further limitation to the use of radioiodinated antibodies is the rapid dissociation (dehalogenation) of the radiolabel from the antibody in circulation (50).

The selection of a radioisotope with a physical half-life matched to the biological half-life of the antibody or fragment can result in the majority of the decay, and hence radioactive emissions, occurring while the greatest quantity of radiopharmaceutical is localized in the tumor. Williams, *et al* have developed formulas termed the imaging figure of merit (IFOM) and therapy figure of merit (TFOM) to determine which isotopes best pair with a series of antibodies and fragments based upon the rate of MAb clearance, isotope half life and emisson track length (51). While this approach does not actually increase the localization of antibodies in tumors, it can be used to optimize the efficiency and specificity of an imaging or treatment system.

Labeling methods. The PIB (52) and SIB (53) methods have been developed to stably label antibodies with radioiodine, such that the resulting conjugates are resistant to dehalogenation in circulation and functionally residualize in tumor cells following internalization. The use of these methods has resulted in significantly greater tumor retention and targeting specificity with intact MAb (52, 54) and scFv-based immunoconjugates (47) and leads to superior retention when the target antigen is internalized.

While radiometals can also be stably, and from a biologic perspective, irreversibly, bound to an antibody molecule if the appropriate chelating agent is employed, natural catabolism of the radiopharmaceutical can result in significant accumulation of the radiometal in cells of the reticulo-endothelial system (*e.g.*, the Kupffer cells in the liver and in the spleen). In the cases where radiometals are employed on antibodies targeting antigens retained on the surface of tumor cells, the use of metabolizable linkers between the chelating agent and the MAb could significantly reduce the retention of radiolabel in catabolic organs, thus increasing the targeting specificity (55).

Pretargeting

A promising technique for overcoming the limitations of antibody-based tumor targeting, without sacrificing the specificity of small rapidly cleared reagents or the quantitative delivery associated with intact IgG molecules, involves multi-step pretargeting processes. Based upon processes pioneered by Hnatowich (56), Paganelli (57) and Goodwin (58), a number of pretargeting protocols, are currently being evaluated. Typically the protocols involve a three step process outlined in Figure 3. Initally, an anti-tumor antibody-streptavidin conjugate is administered and allowed to accumulate in the tumor. After 24-72 hours, a clearing agent (*e.g.*, biotinylated glycosylated albumin or anti-mouse immunoglobulin) is administered to accelerate the hepatic elimination of circulating anti-tumor MAb. Finally, after an additional 24 hours, a cytotoxic agent (*e.g.*, a radioisotope or prodrug) capable of binding to or interacting with the primary antitumor MAb is administered. Axworthy *et al* have used the above strategy to deliver biotinylated ^{90}Y-DOTA to tumors using the NR-LU-10 antibody targeting a pan-carcinoma antigen and have observed complete remissions of established tumors and very impressive tumor:organ ratios in immunodeficient mice (59). The same group has reported highly specific tumor retentions and objective tumor regressions in phase I clinical trials of pretargeting that are currently underway (59,60).

Similar strategies have been employed by a number of investigators for antibody dependent prodrug therapy (ADEPT) of cancer (reviewed in reference 61). In ADEPT, an antibody-enzyme conjugate is administered and allowed to localize to tumor. After actively clearing circulating antibody with secondary antisera or waiting until the majority of the circulating antibody has been eliminated, an inactive prodrug (*e.g.*, etoposide phosphate) is administered which is converted by the enzyme-antibody conjugate to its active, cytotoxic form (*e.g.*, etoposide). Using this technique, highly specific deposition of active drug (tumor:tissue ratios of greater than 8000:1) were reported by Begent *et al* at the keystone Symposia Conference on Exploring and Exploiting Antibody and Ig Super Family combining sites (Taos, NM, 1996).

Summary

Clearly major progress has been made in the development of antibody based strategies for the treatment of cancer. However, the field is still faced with the daunting task of interpreting the diverse results generated with a myriad of constructs targeting a wide variety of antigens.

The point has been reached where it is necessary for a series of rigorous experiments to be performed with a panel of antibody-based species, derived from a single antibody-based molecule, targeting a single antigen epitope. Until this comes to pass, anecdotal results stemming from the use of different tumor models, affinities and valences will limit the validity of any derived conclusions.

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Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site.

Schier R, McCall A, Adams GP, Marshall KW, Merritt H, Yim M, Crawford RS, Weiner LM, Marks C, Marks JD.

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We determined the extent to which additional binding energy could be achieved by diversifying the complementarity determining regions (CDRs) located in the center of the antibody combining site of C6.5, a human single-chain Fv (scFv) isolated from a non-immune phage library which binds the tumor antigen c-erbB-2. CDR3 of the light (V(L)) and heavy (V(H)) chain variable region of C6.5 were sequentially mutated, the mutant scFv displayed on phage, and higher affinity mutants selected on antigen. Mutation of V(L) CDR3 yielded a scFv (C6ML3-9) with a 16-fold lower Kd (1.0 x 10(-9) M) than C6.5. Due to its length of 20 amino acids, four V(H) CDR3 libraries of C6ML3-9 were constructed. The greatest increase in affinity from a single library was ninefold (Kd = 1.1 x 10(-10) M). Combination of mutations isolated from separate V(H) CDR3 libraries yielded additional ninefold decreases in Kd, resulting in a scFv with a 1230-fold increase in affinity from wild-type C6.5 (Kd = 1.3 x 10(-11) M). The increase in affinity, and its absolute value, are comparable to the largest values observed for antibody affinity maturation in vivo or in vitro and indicate that mutation of V(L) and V(H) CDR3 may be a particularly efficient means to increase antibody affinity. This result, combined with the location of amino acid conservation and substitution, suggests an overall strategy for in vitro antibody affinity maturation. In addition, the affinities and binding kinetics of the single-chain Fv provide reagents with potential tumor targeting abilities not previously available.

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Human IgG2 variants of chimeric anti-CD3 are nonmitogenic to T cells.

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The mouse anti-human CD3 mAb OKT3 is a potent immunosuppressive agent used for the treatment of acute transplant rejection. OKT3 therapy is associated with acute toxicity resulting from in vivo T cell activation and systemic cytokine release, and a human anti-mouse Ab response. T cell activation is thought to be triggered by CD3 cross-linking mediated by the Abs bridging T cells and Fc receptor-bearing cells. Recent studies in a mouse model indicate that anti-mouse CD3 Abs with low affinity for Fc receptors can achieve immunosuppression without T cell activation, toxicity, or an anti Ab response. To obtain an analogous Ab to improve the current anti-human CD3 therapy, a humanized Ab with low affinity for Fc receptors is needed. In this study, we introduced mutations into the upper CH2 region of IgG2 and expressed the altered Fc as chimeric OKT3 Abs. Compared with chimeric OKT3 IgG1, IgG2, IgG3, and IgG4, the IgG2 mutants were less mitogenic to T cells, and they did not induce the release of TNF-alpha, IFN-gamma, or IL 2. In parallel, we observed no functional interaction of the IgG2 mutant Abs with K562 cells, which express the IgG2-binding Fc receptor on their surface Despite no measurable T cell activation, the mutant Abs could still modulate the CD3 complex. When coupled to a humanized anti-CD3, the IgG2 variant may provide a drug with less acute toxicity and immunogenicity, but may still retain potent immunosuppressive properties.

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